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Antimicrobial effect and membrane-active mechanism of Urechistachykinins, neuropeptides derived from *Urechis unicinctus*

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Abstract We investigated the antimicrobial effects of Urechistachykinins I and II (UI and UII) and their modes of action. UI and UII showed antimicrobial activities without a hemolytic effect. To investigate the mechanism(s) of UI and UII, cellular localization was examined. Confocal microscopy results showed that peptides were located in the cell envelope. To elucidate the physical changes of membrane induced by UI and UII in *Candida albicans*, flow cytometry analyses were performed by using bis-(1,3-dibutylbarbituric acid) trimethine oxonol, and changes in membrane dynamics were assessed using 1,6-diphenyl-1,3,5-hexatriene. The results suggest that UI and UII may exert their antimicrobial effect by disrupting the cell membranes.

Keywords: Urechistachykinin; Antimicrobial peptide; Neuropeptide; Membrane-active mechanism; *Urechis unicinctus*

1. Introduction

Short cationic amphipathic peptides with antimicrobial activities and/or immunomodulatory activities (antimicrobial peptides) are present in virtually every life form as an important component of innate immune defenses [1,2]. Similarly to antimicrobial peptides, peptides with neural or neuroendocrine signaling functions (neuropeptides) are generally amphipathic molecules, a property that permits them to achieve high local concentrations in the aqueous space between the nerve ending and receptor, as well as the target membrane. This shared property of amphipathicity led to the discovery of antimicrobial activities of many neuropeptides, and some have been shown to have potent antimicrobial activity [3,4]. In *Caenorhabditis elegans*, a conserved family of infection-induced, neuropeptide-like proteins (NLP-27, NLP-28, NLP-29, NLP-30, NLP-31, NLP-33) were found and thought to have neuromodulatory and antimicrobial functions [5]. In a mammalian nervous system, substance P, involved in pain, signal transmission, inflammatory processes and vasodilatation, has an antimicrobial activity [3,6].

If a long list of neuropeptides is examined it is possible to formulate several groups based on the sequence homologies of amino acids that constitute the neuropeptides. One of these groups is the tachykinin superfamily [7]. Some peptides of the tachykinin superfamily, including substance P, contain the consensus sequence – Phe-X-Gly-Leu-Met-NH_2 at their C-termini [8]. Peptides with some sequence similarities to that of the tachykinin superfamily have also been identified from invertebrates. These peptides have been designated tachykinin-related peptides (TRPs) and are characterized by the preserved C-terminal pentapeptide – Phe-X-Gly-Y-Arg-NH_2 (X and Y are variable residues) [9]. UI and UII are examples of TRPs. They were isolated from the ventral nerve cords of the echiuroid worm, *Urechis unicinctus*. These peptides showed a contractile action on the inner circular body-wall muscle of the animal [10]. Similar properties of UI and UII to substance P – they are neuropeptides that have sequence similarities. This leads us to think that UI and UII might have similar antimicrobial activities like substance P.

In this study, antimicrobial activities of UI and UII were measured. We then investigated the antimicrobial mechanism of the UI and UII and suggested a potential for providing a template regarding the design of a potent antimicrobial peptide possessing a novel antimicrobial mechanism.

2. Materials and methods

2.1. Materials

Vancomycin, kanamycin and amphotericin B were purchased from the Sigma Chemical Co. (St. Louis, Mo, USA). Stock solutions of vancomycin and kanamycin were prepared in sterile distilled water and solutions of amphotericn B were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. For all experiments, a final concentration of 1% DMSO was used as the solvent carrier.

2.2. Peptide synthesis

The peptides were synthesized by the solid phase method using 9-fluorenyl-methoxycarbonyl chemistry [11]. The crude peptide was repeatedly washed with diethylether, dried in a vacuum, and purified using a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19 × 30 cm). The purity of the peptide was checked by an analytical reversed-phase HPLC on an Ultrasphere C_{18} column (4.6 × 25 cm; Beckman, Fullerton, CA, USA). The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI-mass spectrometer [12].

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Abbreviations: UI, Urechistachykinin I; UII, Urechistachykinin II; DMSO, dimethyl sulfoxide; MTT, (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide); CLSM, confocal laser scanning microscopy; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; PBS, phosphate buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene

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2.3. Microorganisms and culture conditions

Streptococcus mutans (KCTC 3065), *Trichosporon beigelii* (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecium* (ATCC 29212), *Escherichia coli* O-157 (ATCC 43895), *Pseudomonas aeruginosa* (ATCC 27853), *Vibrio vulnificus* (ATCC 29307) and *Candida albicans* (ATCC 90028) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Bacterial cells were cultured in a Mueller-Hinton broth (Difco, Sparks, MD, USA) with aeration at 37 °C. Fungal cells were cultured in a YPD broth (Difco) with aeration at 28 °C. *M. furfur* was cultured at 32 °C in a modified Bacto yeast extract/ malt extract (YM) broth (Difco) and 1% olive oil.

2.4. Determining of antimicrobial susceptibility

Bacterial cells (2×10^7/ml), inoculated into a Mueller-Hinton broth and 0.1 ml/well, were dispensed in 96-well microtiter plates. MICs were determined by means of a serial 2-fold dilution of test peptides in following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [13]. After 24 h of incubation at 37 °C, the minimal concentration of peptide to prevent the growth of a given test organism was determined and defined as MIC. Growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, CA, USA) by monitoring absorption at 620 nm. Fungal cells (2×10^4/ml) were inoculated into an YPD or YM broth and 0.1 ml/well were dispensed into microtiter plates. MICs were determined by a serial 2-fold dilution of test peptides, following a micro-dilution method and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [14]. After 48 h of incubation at either 28 °C or 32 °C, the minimal concentration of peptide to prevent the growth of a given test organism was determined and defined as MIC. Growth was assayed with a microtiter ELISA Reader by monitoring absorption at 580 nm. MIC values were determined by three independent assays.

2.5. Hemolytic activity against human erythrocytes

The hemolytic activity of the peptides was evaluated by determining the release of hemoglobin from a 4% suspension of fresh human erythrocytes at 414 nm with an ELISA plate Reader [14]. The hemolysis percentage was calculated by using the following equation: Percentage hemolysis = $[(Abs_{414\ nm}$ in the peptide solution $- Abs_{414\ nm}$ in a PBS)/ ($Abs_{414\ nm}$ in 0.1% Triton X-100 $- Abs_{414\ nm}$ in a PBS)] $\times 100$.

2.6. Confocal laser scanning microscopy (CLSM)

The intracellular distribution of peptides was analyzed by means of a CLSM. Cells were treated with FITC-labeled UI or UII and incubated for 5 min at 28 °C. After incubation, cells were harvested by centrifugation at 10000 rpm for 5 min and washed with an ice-cold phosphate buffered saline (PBS [pH 7.4]). Visualization and localization of the labeled peptide were performed by a Laser Scanning Spectral Confocal Microscope (Leica TCS SP2, Leica, Swiss).

2.7. Flow cytometric analysis for the plasma membrane potential

For analysis of the membrane integrity after peptide treatment, log-phased cells of *C. albicans* (1×10^8 cells) were harvested and resuspended with a 1-ml fresh YPD medium, containing UI, UII or melittin (at the MIC) as a positive control. After incubation for 3 h, the cells were washed with a PBS. To detect depolarization of the cell membrane, 1 ml PBS containing 50 μg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Molecular Probes, Eugene, OR, USA), was added and the samples were incubated for 1 h at 4 °C in the dark [15]. Flow cytometric analysis was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.8. Measurement of plasma membrane fluorescence anisotropy

Fluorescence from the plasma membrane of *C. albicans* cells labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes) were detected to investigate changes in membrane dynamics. Fungal cells (1×10^8 cells) were treated with peptides in the concentration range between 0 and MIC values and incubated for 3 h at 28 °C. Labeling and fluorescence measurement were performed, exactly as described [16].

3. Results and discussion

Peptides were chemically synthesized by the solid phase method, and were identified by amino acid analysis and MALDI-mass spectroscopic analysis (Table 1). In this study, melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), a 26-residue peptide which is derived from the venom of the European honey bee *Apismellifera*, was used as reference to compare with UI and UII. Melittin is an antimicrobial peptide with a powerful lytic activity against both bacterial and eukaryotic cells [17]. Additionally, vancomycin, kanamycin and amphotericin B were used as positive controls against Gram-positive bacteria, Gram-negative bacteria and fungi, respectively.

The antibacterial activities of UI and UII against human pathogenic Gram-positive and Gram-negative species of bacteria were determined by using the broth micro-dilution method. The antibacterial activity of UII was stronger than that of UI. However, the antibacterial activities of UI and UII are less potent compared to melittin. Although UI and UII were less active than vancomycin against Gram-positive bacteria, these peptides exhibited 4–8-fold increased activity compared to kanamycin against Gram-negative bacteria. The bacterial strains tested are susceptible to UI and UII with MIC values in the 10.6–42.5 μM range, while melittin is determined at the MIC value of 1.1–4.4 μM (Table 2). The antifungal activities of UI and UII were measured by MTT assay. UI showed strong activity at levels of 21.3–42.5 μM. The fungal strains tested are susceptible to UII with MIC values in the 12.7–50.9 μM range. The antifungal activities of UI and UII are less potent than that of melittin, which is determined at the MIC value of 4.4–8.8 μM. However, UI and UII displayed similar antifungal activities compared to amphotericin B which is determined at the MIC value of 3.4–54 μM (Table 2).

Although most antimicrobial peptides exhibited relatively good cell selectivity, between microbial cells versus mammalian host cells, some antimicrobial peptides show a lytic activity toward mammalian cells. Therefore, we examined the ability of UI and UII in causing the hemolysis of human erythrocytes as a measure of their toxicity to mammalian cells. UI and UII showed no cytotoxicity on human erythrocytes at all tested concentrations, while melittin exhibited a potent hemolytic activity regarding most tested concentration amounts (Table 3). These results demonstrated that UI and UII have high selectivity of antimicrobial activities; in other words, these peptides possess cell selectivity between microbial cells and mammalian cells.

To provide information on the mode of antimicrobial action of UI and UII, we have selected *C. albicans* as a model organism, which is both a commensal and opportunistic pathogen of ever-increasing medical importance [18]. To examine the target sites of UI and UII in *C. albicans*, these were labeled with FITC and visualized under confocal microscopy. FITC-labeled UI and UII accumulated in the cell envelope immedi-

Table 1
Amino acid sequences and molecular masses determined by MALDI-MS of Urechistachykinins

Peptides	Amino acid sequences	Calculated values	Observed values	Retention time (min)
UI	LRQSQFVGSR-NH_2	1176.3	1176.7	12.220
UII	AAGMGFFGAR-NH_2	983.1	982.9	15.842

Table 2
Antimicrobial activities of UI and UII

Microorganisms	MIC (μM)					
	UI	UII	Vancomycin	Kanamycin	Amphotericin B	Melittin
Gram-positive bacteria						
Streptococcus mutans	21.3	12.7	4.2	ND	ND	1.1
Staphylococcus aureus	42.5	25.4	4.2	ND	ND	4.4
Enterococcus faecium	21.3	25.4	8.4	ND	ND	2.2
Gram-negative bacteria						
Escherichia coli O-157	10.6	12.7	ND[a]	10.7	ND	1.1
Pseudomonas aeruginosa	21.3	12.7	ND	172	ND	2.2
Vibrio vulnificus	42.5	25.4	ND	172	ND	4.4
Fungi						
Candida albicans	42.5	25.4	ND	ND	27	8.8
Trichosporon beigelii	21.3	12.7	ND	ND	54	4.4
Malassezia furfur	42.5	50.9	ND	ND	3.4	8.8

[a]ND, Not done.

Table 3
Hemolytic activities of the peptides against human erythrocyte cells

Peptides	% Hemolysis (μM)					
	100	50	25	12.5	6.25	3.12
UI	0	0	0	0	0	0
UII	0	0	0	0	0	0
Melittin	100	100	100	100	100	75

ately after addition to the cells (Fig. 1), suggesting that the major target site of UI and UII is the envelope of the fungal cells.

To assess whether UI and UII affect the function of the fungal plasma membrane, we investigated their ability to dissipate the membrane potential of *C. albicans*. *C. albicans* cells were cultured in the presence or absence of UI, UII or melittin and the amounts of accumulated $DiBAC_4(3)$ in cells with flow cytometry by staining with $DiBAC_4(3)$ were measured. $DiBAC_4(3)$ has a high voltage sensitivity and can enter depolarized cells, where it binds to lipid-rich intracellular components [15]. As shown in Fig. 2, similarly to melittin, which is known as a membrane-acting antimicrobial peptide, UI or UII-treated cells caused a higher accumulation of $DiBAC_4(3)$ than peptide-

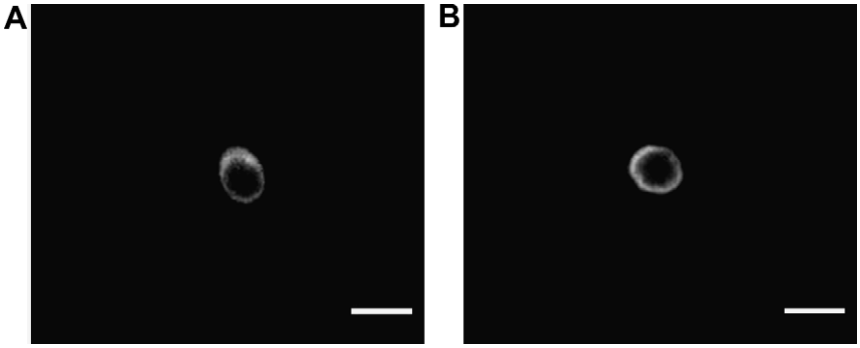

Fig. 1. Confocal fluorescence microscopy of *Candida albicans* cells treated with FITC-labeled peptides: (A) treated with UI and (B) treated with UII.

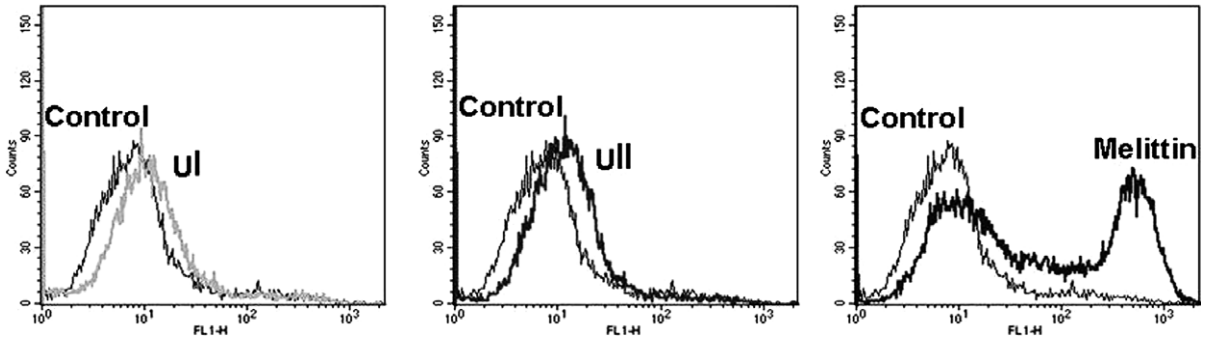

Fig. 2. FACScan analysis of $DiBAC_4(3)$ staining in *Candida albicans*. Histograms showed the fluorescence intensity of stained $DiBAC_4(3)$ after *C. albicans* were treated with peptides.

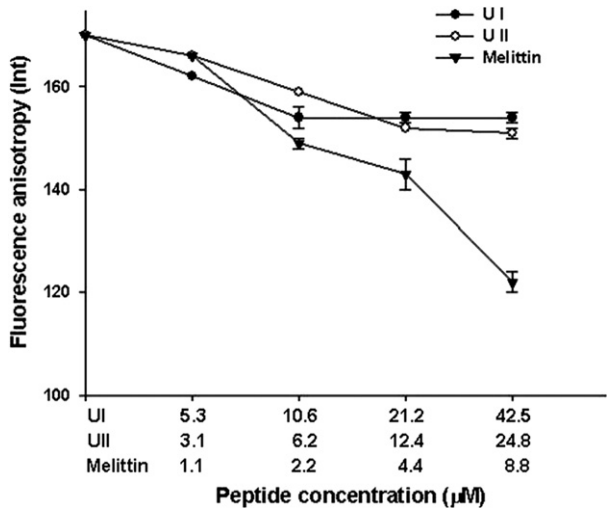

Fig. 3. DPH fluorescence anisotropy after the addition of peptides. Treatment with UI, UII, or melittin in the concentration range between 0 and MIC values, and the error bars represent the standard deviation (SD) values for three independent experiments, performed in triplicate.

untreated cells. These results indicate that UI and UII affect yeast cells by injuring their membranes, thus disrupting membrane potential.

The effects of UI and UII on the fungal plasma membrane were further investigated with a fluorescent membrane probe. DPH is a hydrophobic molecule and this property makes it possible to associate with the hydrocarbon tail region of phospholipids within the cytoplasmic membrane without disturbing the structure of the lipid bilayer [19]. Assuming the antifungal activities by UI and UII on *C. albicans* are exerted at the level of the plasma membrane, DPH, which interacts with an acyl group of the plasma membrane lipid bilayer, would not be inserted into the membrane. The results showed that the DPH fluorescence anisotropy of UI or UII-treated cells decreased in a dose-dependent manner, but less so than in regards to the decrease of fluorescence anisotropy in the presence of melittin (Fig. 3). This suggested that the plasma membrane is structurally disrupted when exposed to UI or UII. As for the mechanism by which they break down the membrane permeability barrier, it is possible that UI and UII perturb the membrane lipid bilayers, causing the dissipation of the electrical potential of the membrane. Although the exact mechanism of the actions of UI and UII has not as yet been fully elucidated, the results reported here indicate their effects on the plasma membrane. Therefore, it is concluded that UI and UII have considerable antimicrobial activity, thus further investigation for clinical applications is required.

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References

[1] Hancock, R.E. and Sahl, H.G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24, 1551–1557.
[2] Brogden, K.A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3, 238–250.
[3] Brogden, K.A., Guthmiller, J.M., Salzet, M. and Zasloff, M. (2005) The nervous system and innate immunity: the neuropeptide connection. Nat. Immunol. 6, 558–564.
[4] Hansen, C.J., Burnell, K.K. and Brogden, K.A. (2006) Antimicrobial activity of Substance P and Neuropeptide Y against laboratory strains of bacteria and oral microorganisms. J. Neuroimmunol. 177, 215–218.
[5] Couillault, C., Pujol, N., Reboul, J., Sabatier, L., Guichou, J.F., Kohara, Y. and Ewbank, J.J. (2004) TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. Nat. Immunol. 5, 488–494.
[6] Kowalska, K., Carr, D.B. and Lipkowski, A.W. (2002) Direct antimicrobial properties of substance P. Life Sci. 71, 747–750.
[7] Hoyle, C.H. (1998) Neuropeptide families: evolutionary perspectives. Regul. Pept. 73, 1–33.
[8] Kawada, T., Satake, H., Minakata, H., Muneoka, Y. and Nomoto, K. (1999) Characterization of a novel cDNA sequence encoding invertebrate tachykinin-related peptides isolated from the echiuroid worm, *Urechis unicinctus*. Biochem. Biophys. Res. Commun. 263, 848–852.
[9] Nässel, D.R. (1999) Tachykinin-related peptides in invertebrates: a review. Peptides 20, 141–158.
[10] Ikeda, T., Minakata, H., Nomoto, K., Kubota, I. and Muneoka, Y. (1993) Two novel tachykinin-related neuropeptides in the echiuroid worm, *Urechis unicinctus*. Biochem. Biophys. Res. Commun. 192, 1–6.
[11] Merrifield, R.B. (1986) Solid phase synthesis. Science 232, 341–347.
[12] Jungblut, P. and Thiede, B. (1997) Protein identification from 2-DE gels by MALDI mass spectrometry. Mass Spectrom. Rev. 16, 145–162.
[13] Clinical and Laboratory Standards Institute (2005) Performance standards for antimicrobial susceptibility testing, fifteenth informational supplement, approved standard MS100-S15, CLSI, Wayne, PA.
[14] Sung, W.S., Lee, I.-S. and Lee, D.G. (2007) Damage to the cytoplasmic membrane and cell death caused by lycopene in *Candida albicans*. J. Microbiol. Biotechnol. 17, 1797–1804.
[15] Liao, R.S., Rennie, R.P. and Talbot, J.A. (1999) Assessment of the effect of amphotericin B on the vitality of *Candida albicans*. Antimicrob. Agents Chemother. 43, 1034–1041.
[16] Lee, D.G., Park, Y., Kim, P.I., Jeong, H.G., Woo, E.-R. and Hahm, K.-S. (2002) Influence on the plasma membrane of *Candida albicans* by HP (2–9)-magainin 2 (1–12) hybrid peptide. Biochem. Biophys. Res. Commun. 297, 885–889.
[17] Blondelle, S.E. and Houghten, R.A. (1991) Hemolytic and antimicrobial activities of the twenty-four individual omission analogues of melittin. Biochemistry 30, 4671–4678.
[18] Klein, R.S., Harris, C.A., Small, C., Moll, B., Lesser, M. and Friendland, G.H. (1984) Oral candidiasis in high risk patients as the initial manifestation of the acquired immunodeficieny syndrome. New Engl. J. Med. 311, 354–358.
[19] Vincent, M., England, L.S. and Trevors, J.T. (2004) Cytoplasmic membrane polarization in Gram-positive and Gram-negative bacteria grown in the absence and presence of tetracycline. Biochim. Biophys. Acta 1672, 131–134.